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The Effects of Ginseng Saponin Fraction on Growth and Siderophore Formation in *Escherichia coli K-12*

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인삼사포닌 분획이 *Escherichia coli* K-12의 성장과 Siderophore 생성에 미치는 영향

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Abstract

The effects of saponin, one of major components (*Panax ginseng* C.A. Meyer), on the growth of *E. coli* K-12 and the formation of siderphore was observed The following results were obtained.

1. When *E. coli* was grown on medium containing $1\times10^{-5}\%$—$1\times10^{-1}\%$ of the saponin, the rate of growth was stimulated at $10^{-1}\%$ of the saponin significantly compared to that of control.
2. When *E. coli* K-12 was grown on medium containing $1\times10^{-1}\%$ of the saponin, the amount of siderphore was two times as much as the control.
3. The growth of *E. coli* was observed to be dependent on the concentration of siderophore when siderophore was added to medium.
4. The effect of saponin on the formation of siderophore *in vitro* was observed to reach maximum at $1\times10^{-3}\%$ of the saponin.

Such results suggest that the growth rate of *E. coli* K-12 could be enhanced by ginseng saponin fraction through stimulation of siderphore formation.

We have described the fast growth of *E. coli,* K-12 and B. subtilis, rapid uptake of ^{14}C-glucose, and high level of other metabolites such as lipids and proteins of *E. coli,* and *B. subtilis* in medium containing saponing fraction compared to that of microorganisms without saponin fraction.[1~3] Such differences were claimed to be due to rapid uptake of ^{14}C-glucose by widened periplasmic region through unknown mechanism in the prescence of saponin fraction in medium[3] and have raised a question whether there is another possible factor, siderophore[4] (Greek for iron bears), since microorganisms must secure a sufficient amount of iron for normal growth. These are known to be synthesized by the cells under iron-deficient condition and in most case, excreted into the medium[5], where they can complex and solubilize any iron present there. It is generally believed that these complexes are then taken into the cells presumably by specific transport systems, thus providing iron for cell metabolism. Within the group of enteric

bacteria, only three species (*E. coli, S. typhimurium,* and *A. aerogense*) have, so far, been studied in any detail. The main iron-binding compound produced by these species is enterochelin, and its role in iron transport is now well established. And biosynthesis of enterochelin from 2, 3-dihydroxybenzoate and serine in the prescence of magnesium ions and ATP was reported[6]. 2, 3-dihydroxybenzoate was also shown to involve isochorismate and 2, 3-dihydro-2, 3-dihydroxybenzoate as intermediate.[7~11]

The present paper deals with the effect of ginseng saponin fraction on growth, the level of enterochelin formation *in vivo* and the conversion of 2, 3-dihydroxybenzoate and serine into entrochelin *in vitro,* and entrochelin obtained on the growth in relation to possible explanation of ginseng saponin fraction on the rapid growth of *E. coli,* K-12.

Materials and Method

1. Bacterial strain

The strain used was tryptophan auxotroph derived from it. It was obtained from Dr. S.Y. Lee, Department of Agricultural chemistry, Korea University.

2. Chemicals

The following chemicals were obtained from commercial source: 2, 3-dihydroxybenzoate (Tokyo Kasei Kogyo Co.), serine, dithiothreitol and ATP (Sigma Chemical Co.), the others (E. Merck, and Aldrich Chemical Co.).

3. Growth medium

The minimal medium without added iron element, was used for all experiments. It contained, in grams per liter, Na_2NPO_4, 8.2; KH_2PO_4, 2.7; $(NH_4)_2SO_4$, 1; $MgSO_4$, 0.1; $Ca(NO_3)_2$, 0.005; glucose, 5; thereonine, 0.04; leucine, 0.02; tryptophan 0.02; thiamin, 0.001. The removal of iron from medium was made by combined methods[12,13]. The amount of iron was routinely checked by usage of bathophenanthroline as described[9].

4. Growth of bacteria

Cultures were grown in 50ml volumes in 500ml pyrex Erlenmyer flasks, which were incubated in a shaking water bath set 250rpm and 37°C. Bacterial growth was followed by measuring the turbidity of the cultures at 600nm with colman Junior II Spectrophotometer.

5. Assay of siderophore in medium

Saponin concentrations ($10^{-5}\%-1\times10^{-1}\%$) were added to different Erlenmyer flask containing iron free medium. Supernatants were obtained from late exponentiol phase by centrifugation at 6,000xg for 15min. The amount of siderophore in the cell free supernatant was determined as described elsewhere[14]. Ferric siderophore was also prepared by adding 0.5ml ferric iron solution (9.5mg/ml) to 2ml cell free supernatant as used[15]. Ferric siderophore was applied to DEAE-cellulose column (2×20cm) previously equilibriated with 10mM phosphate buffer (pH, 7.1). Ferric siderophore was collected by using step gradient; the same buffer containing NH_4Cl 0-0.2M, 0.2-2M, and 2M—5M, respectively. The each elution volume was 20ml/20min. Only reddish fractions were pooled, acidified to pH 1.2. and extracted with ethylacetate. Ethylacetate in the extract was removed by rotatory evaporator as described[16-19]. The identification of siderophore was checked as followings[20]. Two-dimensional thin-layer chromatography was carried out on cellulose sheet (E. Merck. 5577) using benzene-acetic acid-water (125:72:3, by vol.) solvent for the first dimension.[27] After drying, the plate was developed in the second dimension using 5% (w/v) ammonium formate in 0.5% (v/v)

aqeous formic acid. Siderophore was detected by spraying with 0.5% (w/v) $FeCl_3$.

6. The effect of siderophore on growth

Purified siderophore was sterilized by using membrane filter (0.45um pore size). and added to medium (final siderophore concentration; 1×10^{-4}M, 5×10^{-5}M, and 1×10^{-5}, and 1×10^{-6}M) The effect of siderophore on growth was checked.

7. Biosynthesis of enterochelin in vitro

Cells excreted enterochelin into medium were washed by 0.1M phosphate butter (plf 7.1). 1gr washed cells suspended in 5ml 0.1M Tris-HCl (pH, 8.0) containing 5mM periplasmic were disrupted by sonicator (Lab-Line Instrument, Inc., U.S.A.) at 0°~4°C. The homogenate was centrifuged at 20,000xg for 30min at 4°C, and supernatant was used as enzyme source responsible for synthesizing enterochelin as described elsewhere[21~24]. The incubation mixture employed to measure the synthesis of enterochelin contained, in a total bolume of 1.2ml, 0.5ml of enzyme (pH, 8.0), 1*u*mole of 2, 3-dihydroxybenzoate, 1.5*u* moles of L-serin, and 10*u* moles of ATP in 0.7ml Tris buffer (pH, 8.0). In case of observing ginseng saponin effect on enzyme activity, the total volume also was same. The reaction mixture was incubated for 60min at 37°C, and the reaction was stopped by acidifying to pH, 1.2 with 1N HCl. Enterochelin was extracted with 4.8ml ethylacetate and ethylacetate was completely removed by rotatory evaporator. Thin-layer chromatography of residue dissolved in small amount of ethylacetate was carried out on cellulose sheet using benzene-acetic acid-water (125:72:3, by vol.) solvent. Spots were detected by TLC-scanner (Model CS-910 Shimadzu).

Results and Discussion

The growth rates of *E. coli*, K-12 in medium containing the saponin fraction ranging from 10^{-5}% to 10^{-1}% were observed and 10^{-1}% was the best whereas the other was more or less compared to the control. The growth rate for 10^{-1}% was only shown in Fig. 1. At late exponential phase, there was a big increase in *E. coli*, K-12 grown in medium containing 10^{-1}% saponin. Such trends are in good agreement with previous

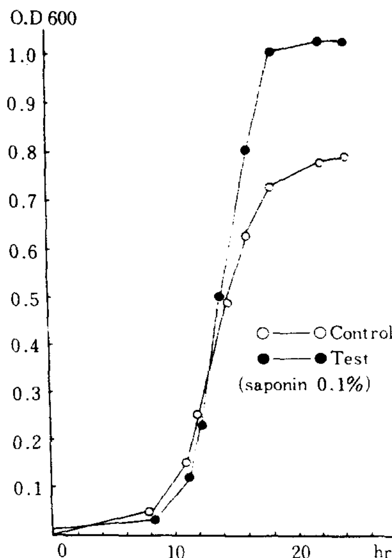

Fig. 1. Growth curve of *E. coli* K-12 grown in medium with and without ginseng saponin fraction.

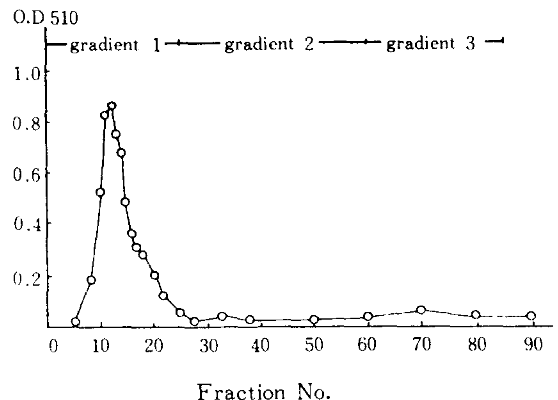

Fig. 2. Elution profiles of siderophore on DEAE-cellulose column.

Table 1. The dependence of siderophore formation on concentration on saponin fraction

concentration of saponin fraction (%)	0	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
O.D. at 600mm (growth)	0.80	1.10	0.90	0.70	0.90	0.98
concentration of siderophore (*u*M)	32	110	50	20	31	35
conc. of sidero-phore/O.D. value	*100	250	140	73	85	90

* The 100 was arbitrarily given to the control.

report[25].

As shown in Table 1, the remarkable increase in siderophore amount was observed in cells grown in medium containing 10^{-1}% ginseng saponin fraction. The rest was almost same with the control. Such results suggest that the fast growth rate at 10^{-1}% saponin fraction could be due to increase in amount of siderophore. Rapid uptake of ^{14}C-glucose and widened periplasmic region of *E. coli* grown in medium containing saponin fraction[3] and present results could explain the role of ginseng saponin fraction as far as the increase in growth rate of microorganism is concerned.

As shown in fig. 2, gradient 1 (0-0.2M) was observed to contain reddish ferric siderophore whereas the other gradient was shown to have not. The scanning pattern of siderophore was in an good agreement with previous reports[17]. Identity of siderophore was also checked by two-dimensional chromatography and shown to be same with previous result[20]. The amount of siderophore excreted into medium was observed to be high at late exponential phase.

As shown in Fig. 3, the growth rate of *E. coli,* K-12 was shown to be proportional to the amount of

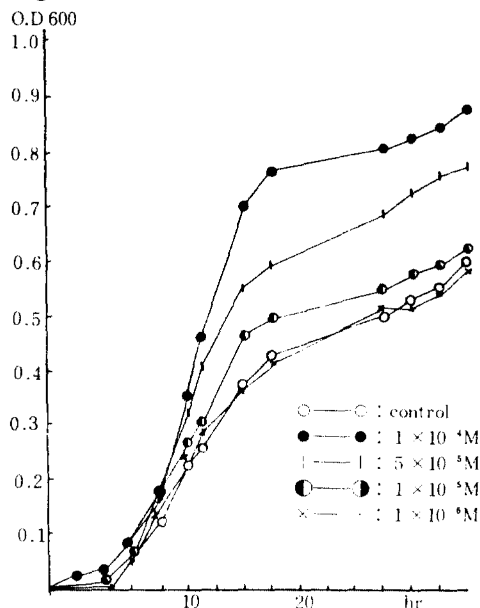

Fig. 3. Effect of sidorophore on growth of *E. coli* K-12.

siderophore obtained from medium. Except 1×10^{-6}M of siderophore, the rest concentrations were observed to increase the rate of growth. Therefore siderophore obtained must have biological activity.

As shown in Table 2, saponin fraction was observed to increase the enterochelin synthesis *in vitro,* remarkably at 10^{-3}%. The rest was almost similar. The effect of saponin fraction on microorganism enzymes[1] was observed. However, the mechanism was not clarified. As far as hydrophobic region of saponin molecule is concerned, saponin may change enzyme conformation as same pattern suggested[26]. Anyhow, saponin fraction seems to have effect on enzyme considering the uptake of saponin in *E. coli*[25], and *P. aeruginosa*[28] grown in medium containing saponin.

Table 2. The dependence of siderophore formation on concentration of saponin fraction *in vitro.*

concentration of saponin (%)	0	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
amounts of siderophore	*100	133	143	157	100	110

* The 100 was arbitrarily given to the control.

요 약

본 실험에서는 한국산 인삼(Panax ginseng C. A. Meyer)의 중요 성분군의 하나인 인삼saponin 분획이 미생물(*E. coil* K-12)의 성장과 siderophore 형성에 미치는 영향을 관찰하여 다음과 같은 결과를 얻었다.

1. 인삼 saponin을 1×10^{-5}%~1×10^{-1}%의 농도로 첨가한 배지에서 *E. coil* K-12를 배양하였을때 농도가 높아짐에 따라 증식이 촉진되었으며 1×10^{-1}%에서는 대조군에 비해 증식이 현저히 촉진되었다.
2. 인삼 saponin 농도 1×10^{-1}%에서 자란 *E. coli* K-12의 siderophore 형성은 대조군에 비해 2배 증가하였다.
3. 정제된 siderophore의 농도에 따른 *E. coli* K-12의 증식은 배지내의 siderophore 농도가 증가함에 따라서 증가되었다.
4. siderophore의 *in vitro* 생합성에 대한 인삼 saponin의 효과를 관찰한 결과 인삼 saponin 농도 1×10^{-3}%에서 최고값을 나타내었다.

따라서 인삼 saponin은 siderophore의 형성을 촉진시켜 *E. coil* K-12의 증식을 빠르게 하는것 같다.

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